EXAMPLE 25

Primer-Directed And Primer Independent Cleavage
Occur At The Same Site When The Primer Extends To
The 3' Side Of A Mismatched "Bubble" In The Downstream Duplex

As discussed above in Example 1, the presence of a primer upstream of a bifurcated duplex can influence the site of cleavage, and the existence of a gap between the 3' end of the primer and the base of the duplex can cause a shift of the cleavage site up the unpaired 5' arm of the structure (see also Lyamichev *et al., supra* and U.S. Patent No. 5,422,253). The resulting non-invasive shift of the cleavage site in response to a primer is demonstrated in Figures 9, 10 and 11, in which the primer used left a 4-nucleotide gap (relative to the base of the duplex). In Figures 9-11, all of the "primer-directed" cleavage reactions yielded a 21 nucleotide product, while the primer-independent cleavage reactions yielded a 25 nucleotide product. The site of cleavage obtained when the primer was extended to the base of the duplex, leaving no gap was examined. The results are shown in Figure 64 (Figure 64 is a reproduction of Figure 2C in Lyamichev *et al.* These data were derived from the cleavage of the structure shown in Figure 6, as described in Example 1. Unless otherwise specified, the cleavage reactions comprised 0.01 pmoles of heat-denatured, end-labeled hairpin DNA (with the unlabeled complementary strand also present), 1 pmole primer [complementary to the 3' arm shown in Figure 6 and having the sequence: 5'-GAAT TCGATTTAGGTGACACTATAGAATACA (SEQ ID NO:64)] and 0.5 units of DNAP*Taq* (estimated to be 0.026 pmoles) in a total volume of 10 µl of 10 mM Tris-Cl, pH 8.5, and 1.5 mM $MgCl_2$ and 50 mM KCl. The primer was omitted from the reaction shown in the first lane of Figure 64 and included in lane 2. These reactions were incubated at 55°C for 10 minutes. Reactions were initiated at the final reaction temperature by the addition of either the $MgCl_2$ or enzyme. Reactions were stopped at their incubation temperatures by the addition of 8 µl of 95% formamide with 20 mM EDTA and 0.05% marker dyes.

Figure 64 is an autoradiogram that indicates the effects on the site of cleavage of a bifurcated duplex structure in the presence of a primer that extends to the base of the hairpin duplex. The size of the released cleavage product is shown to the left (*i.e.*, 25 nucleotides). A dideoxynucleotide sequencing ladder of the cleavage substrate is shown on the right as a marker (lanes 3-6).

These data show that the presence of a primer that is adjacent to a downstream duplex (lane 2) produces cleavage at the same site as seen in reactions performed in the absence of the primer (lane 1) (*see* Figures 9A and B, 10B and 11A for additional comparisons). When the 3' terminal nucleotides of the upstream oligonucleotide can base pair to the template strand but are not homologous to the displaced strand in the region immediately upstream of the cleavage site (*i.e.*, when the upstream oligonucleotide is opening up a "bubble" in the duplex), the site to which cleavage is apparently shifted is not wholly dependent on the presence of an upstream oligonucleotide.

As discussed above in the Background section and in Table 1, the requirement that two independent sequences be recognized in an assay provides a highly desirable level of specificity. In the invasive cleavage reactions of the present invention, the invader and probe oligonucleotides must hybridize to the target nucleic acid with the correct orientation and spacing to enable the production of the correct cleavage product. When the distinctive pattern of cleavage is not dependent on the successful alignment of both oligonucleotides in the detection system these advantages of independent recognition are lost.

EXAMPLE 26

Invasive Cleavage And Primer-Directed Cleavage When There Is Only Partial Homology In The "X" Overlap Region

While not limiting the present invention to any particular mechanism, invasive cleavage occurs when the site of cleavage is shifted to a site within the duplex formed between the probe and the target nucleic acid in a manner that is dependent on the

presence of an upstream oligonucleotide which shares a region of overlap with the downstream probe oligonucleotide. In some instances, the 5' region of the downstream oligonucleotide may not be completely complementary to the target nucleic acid. In these instances, cleavage of the probe may occur at an internal site within the probe even in the absence of an upstream oligonucleotide (in contrast to the base-by-base nibbling seen when a fully paired probe is used without an invader). Invasive cleavage is characterized by an apparent shifting of cleavage to a site within a downstream duplex that is dependent on the presence of the invader oligonucleotide.

A comparision between invasive cleavage and primer-directed cleavagem may be illustrated by comparing the expected cleavage sites of a set of probe oligonucleotides having decreasing degrees of complementarity to the target strand in the 5' region of the probe (*i.e.*, the region that overlaps with the invader). A simple test, similar to that performed on the hairpin substrate above (Ex. 25), can be performed to compare invasive cleavage with the non- invasive primer-directed cleavage described above. Such a set of test oligonucleotides is diagrammed in Figure 65. The structures shown in Figure 65 are grouped in pairs, labeled "a", "b", "c", and "d". Each pair has the same probe sequence annealed to the target strand (SEQ ID NO:65), but the top structure of each pair is drawn without an upstream oligonucleotide, while the bottom structure includes this oligonucleotide (SEQ ID NO:66). The sequences of the probes shown in Figures 64a-64d are listed in SEQ ID NOS:43, 67, 68 and 69, respectively. Probable sites of cleavage are indicated by the black arrowheads. (It is noted that the precise site of cleavage on each of these structures may vary depending on the choice of cleavage agent and other experimental variables. These particular sites are provided for illustrative purposes only.)

To conduct this test, the site of cleavage of each probe is determined both in the presence and the absence of the upstream oligonucleotide, in reaction conditions such as those described in Example 19. The products of each pair of reactions are then be compared to determine whether the fragment released from the 5' end of the probe increases in size when the upstream oligonucleotide is included in the reaction.